ISOLATION AND PARTIAL CHARACTERIZATION OF TWO HEMORRHAGIC FACTORS FROM THE VENOM OF *LACHESIS MUTA STENOPHRYS*

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ABBREVIATIONS

B.	-	Bothrops
BAEE	-	N-benzoyl-L-arginine ethylester
C.	-	Crotalus
C'	-	serum complement
CMS	-	carboxymethyl sephadex
DEAE	-	diethylaminoethyl
EDTA	-	ethylenediaminetetraacetic acid
Hb	-	hemoglobin
im	-	intramuscular
ip	-	intraperitoneal
L.	-	Lachesis
LD_{50}	-	lethal dose for 50% of animals tested
MW	-	molecular weight
PSS	-	physiologic saline solution
SDS	-	sodium dodecyl sulfate
cm	-	centimeter
g	-	gram
mamps	-	milliamps
mg	-	milligram
ml	-	milliliter
nm	-	nanometer

CHAPTER I

INTRODUCTION

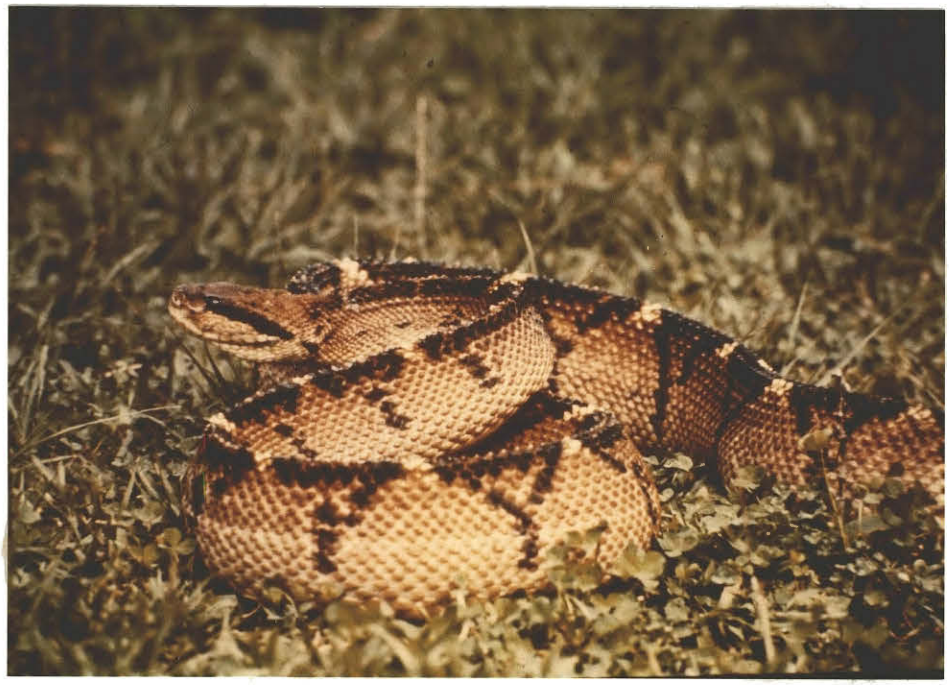
In Costa Rica, snakebite is a relatively common although not always fatal event. Of the 12 known species of toxic snakes found there, 9 belong to the genus Bothrops while Lachesis is represented by only one specie, L. muta, the "bushmaster". This snake occupies the low-lying coastal regions in Costa Rica, but its geographical distribution extends from the south of Nicaragua to the southern border of Brazil. According to Hoge and Romano (1), there are three subspecies of the bushmaster; L. muta stenophrys being the snake found in Costa Rica and Panama.

It is the largest venomous snake in Central and South America, reportedly reaching 12 feet in length with hollow fangs measuring up to 1½ inches in length. The bushmaster is confined to the moist tropics due to thermal requirements demanded by its large size and oviparous method of reproduction. This egg laying feature of L. muta is unique among New World pit vipers and may be a factor in its limited distribution.

Despite the extreme size and the large quantity of venom that can be extracted from the bushmaster, it is thought to be responsible for few incidents of snakebite fatality. As a result, the most studied venoms of Costa Rican fauna belong to the more deadly Bothrops genus. The purpose of this study was to isolate the toxic components of L.

muta venom, and then assay these compounds for certain biological activities. This information will serve as a background for better understanding of bushmaster venom as well as its relation to other Costa Rican venoms (Figure 1).

Figure 1. Bushmaster, Lachesis muta. (Picture from the files of the Instituto Clodomiro Picado.)



CHAPTER II

LITERATURE REVIEW

Since prehistoric times, humans have had contact with venomous snakes and as a result have also suffered from the problems of snakebite. Today, there still is a high incidence of snakebite in many parts of the world. According to Swaroop and Grab (2), 30,000 to 40,000 deaths occur annually worldwide due to snakebite from venomous snakes. These figures may be less than the actual total since some cases of snakebite go unrecorded in underdeveloped countries.

Table I shows the mortality rate due to various venomous species found in Central and South America as compiled by da Fonseca (3).

In an investigation by Rosenfeld (4, 5) of 730 cases of snakebite in Brazil, Bothrops jararaca was responsible for 87% of the bites, followed by Crotalus durissus terrificus with 10%. The remainder was due to various other Bothrops species. However, in Costa Rica Bothrops atrox (asper) causes 80 to 90% of the cases of snakebite with the remaining percent divided among the other 11 venomous snakes.

In these Latin American countries, there is a great need for a simple clinical means that will allow for diagnosis and treatment of the bite without identification of the species. At present, this problem is being combatted by two different methods. The first and most direct is the production of polyvalent antivenins. The second method is the scientific investigation into the composition of the

venoms. The long range goals of this type of research are to understand the biochemical nature of the venom components, how they function and the interrelationships between components.

TABLE I

VENOMOUS SNAKE SPECIES AND FREQUENCY OF BITES*

Species	Number of cases	Percent	Deaths	
			Number	Percent
Crotalus sp.	738	11.18	90	55.9
Bothrops alternatus	384	5.82	8	4.9
Bothrops atrox	83	1.26	1	0.6
Bothrops cotiara	96	1.45	1	0.6
Bothrops jararaca	3446	52.20	25	15.5
Bothrops jararacussu	657	9.95	11	6.8
Bothrops lansbergii	1	0.02	0	0
Bothrops neuwiedi	236	3.58	1	0.6
Bothrops schlegelli	3	0.05	1	0.6
Lachesis muta	16	0.24	1	0.6
Micrurus sp.	15	0.23	0	0
Not specified	926	14.03	22	13.7

*Compiled by da Fonseca (3) for Central and South America.

Snake venoms are viscous secretions, usually yellow or orange in color, and have 25 to 40% dry residue after desiccation (5). Proteins account for up to 90% of the residue, while lipids, peptides, salts, free amino acids and inorganic matter account for the rest (6). The lethal properties of venoms are due to enzymatic as well as non-enzymatic proteins. This lethality can be seen as a 1) neurotoxic response, in which neural transmission is blocked, 2) hemotoxic

response, in which the normal functioning of the circulatory system is disrupted, or 3) myotoxic response, in which necrosis of muscle cells occurs.

Venoms of the Crotalidae family are characterized by the production of hemorrhage, myonecrosis, hypotension and incoagulability of the blood in their victims (6). This can be attributed to a variety of protein components rather than to a single major toxin. As an example of the complexity of Crotalid venoms, Kaiser and Michl (7) report the fractionation of 5 Bothrops species into as many as 14 bands per venom by starch gel electrophoresis. They also report the location of certain enzymatic activities such as phospholipase A, L-amino acid oxidase, phosphoesterases and proteases within these bands. Deutsch and Diniz (8), looking at the proteolytic activities of 15 species of Crotalus, Bothrops and Lachesis venoms, found that all had kinin releasing ability. They also report varying levels of proteolytic digestion of Hb and BAEE, yet found no direct relationship between the two activities. This suggests the presence of several proteases in these venoms.

While there has been no reported isolation of toxic components from bushmaster venom, some of its enzymatic properties have been investigated. Birdsey et al. (9) have shown the ability of L. muta venom to consume hemolytic C' in fresh guinea pig serum. The venom appears to form a stable intermediate with serum factors which is capable of depleting complement components C3-C9 in the presence of EDTA. They also show the isolation of the C' consuming principle of the venom by DEAE cellulose. This factor corresponded to a MW of 17,000 daltons when passed through a Sephadex G-75 column, but it was

unable to induce C' depression upon *in vivo* injection.

Yarleque and Campos (10, 11) have shown the presence of a phosphodiesterase in whole bushmaster venom that has an optimum pH of 9.0, and is activated by mM concentrations of Mg^{+2} and Ca^{+2}. In a second paper, they found 5'-nucleotidase activity in whole venom with peak activity at pH 7.8 and 9.8. Mg^{+2} was found to activate the nucleotidase while Ca^{+2} was inhibitory.

The work of Magalhaes et al. (12) shows the separation and purification of 3 proteases from *L. muta* venom that are active on casein, fibrinogen and bradykinin. Interestingly, bushmaster venom is also known for its strong bradykinin releasing activity (13). Once released from plasma, bradykinin produces strong dilation of the blood vessels, hypotension and smooth muscle contraction. Although it does not have much lethal activity, bradykinin may contribute to rapid immobilization of prey by inducing muscle paralysis.

In their study on 10 venomous snakes from Costa Rica, Guiterrez and Chaves (14) found that the hemorrhagic activity of *L. muta* venom in white mice to be extensive, surpassed only by *B. picadoi* and *B. godmani*. They also report slight myonecrotic activity as well as moderate proteolytic activity for bushmaster venom.

Because of its ability to convert fibrinogen to fibrin, *L. muta* venom is considered to show thrombin-like activity (15). While it has been shown that heparin prolongs the clotting time induced by viperid venoms, Nahas et al. found that thrombin-like venoms such as *L. muta* were not influenced by the anticoagulant effect of heparin. Hence, there is little support for the therapeutic use of this compound in the defibrination syndrome induced by these venoms. Furthermore, the

hemorrhage produced by envenomation appears to be due to other toxic effects of the venom rather than to the incoagulability (16).

Early reports on antivenins expressed the belief that their ability to neutralize the toxic properties of venoms could also be the same as the ability to neutralize the coagulant properties of venoms. However, as early as 1937 investigators have indicated a lack of correlation between the anti-lethal action and neutralization of venom coagulant activity (17). Neutralization by antivenins was also once considered specific for the venoms used in their preparation. Cesari and Boquet (18) state that almost all venoms could be neutralized by heterologous antivenins.

This view was partially confirmed by Klobusitzky and Konig (19) when they observed that Bothrops jararaca and Lachesis muta antivenins neutralized the coagulant fraction of Bothrops jararaca venom. In their study on the cross neutralization of coagulant activity of 27 Crotalid venoms, Rosenfeld and Kelen (20) found a high index of neutralization within venoms of the same genus but no strict specificity between venom and antivenins. L. muta venom was most effectively neutralized by C. durissus terrificus and L. muta antivenins, followed by B. jararaca and then a Bothropic polyvalent antivenin. The L. muta antivenin was most effective against C. durissus terrificus, B. atrox (asper) and C. durissus durissus venoms, and moderately neutralized B. jararaca venom.

Thus, it is apparent that the antigenic composition of the coagulant factors in these venoms have several features in common. This is important not only in combatting the effects of envenomation, but also in establishing the phylogenetic relationships between species.

The toxicity of bushmaster venom has been established by a number of authors, and is usually reported as the amount that kills 50% of the animals inoculated with the venom (21, 22). The LD_{50} for L. muta venom in white mice ranges from 4.51 to 5.59 µg/g mouse by intravenous injection, and from 6.41 to 6.47 µg/g mouse by intraperitoneal route. Another designation of lethality is the Minimum Lethal Dose or the smallest amount of venom just sufficient to cause death. Schottler (23) reports the subcutaneous MLD for L. muta venom to be 6.0 µg/g mouse, while Kaiser and Michl (7) give a subcutaneous value of 4.0 µg/g rabbit. In discussing lethal doses of snake venoms, Klobusitzky (24) presents a table of MLD values of L. muta venom for the dog, rabbit, guinea pig and pigeon by either iv or im injection. These values range from 0.07 mg/kg in the pigeon (iv) to 13-16.5 mg/kg in the guinea pig (im).

This toxicity data will be used to isolate the lethal factors in bushmaster venom so that the biochemical properties of the toxins can be determined.

CHAPTER III

MATERIALS AND METHODS

Venom

One gram of freeze dried L. muta stenophrys (Atlantico) venom and one vial of Bothrops, Crotalus and Lachesis polyvalent antivenin were received as a gift from Dr. Róger Bolaños, Director, Instituto Clodomiro Picado, San Jose, Costa Rica.

Chromatographic Supplies and Animals

Sephadex G-100 and CM Sephadex were purchased from Pharmacia Co. SDS, acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED) and N,N'-methylenebisacrylamide (Bis) were from the Sigma Chemical Co. 2-Amino-2-hydroxymethyl-1,3-propanediol (Tris) and bromophenol blue were purchased from Canalco. Ammonium persulfate and glycine were from Eastman Kodak. Aniline blue black and coomassie blue were from Polysciences Inc. Agar was from Difco Laboratories, CD1 line white mice were purchased from Holtzman Co.

Gel Filtration

Dry Sephadex G-100 was allowed to swell in 2 to 3 times the final bed volume of water and stored at 4 C for 72 hours. The excess water was decanted and the gel was packed into a 2.5 x 90 cm glass column

over a period of 3 hours. The column was equilibrated by washing with at least twice the column volume of distilled water. The Sephadex column was calibrated using 0.1 ml of a standards solution containing milligram quantities of proteins with known molecular weights. The flow rate was kept at 0.6 ml/min and 3 ml fractions were collected to give at least 85 fractions. Absorbance of the samples was measured with a Coleman 101 spectrophotometer at 280 nm.

For venom fractionation, 0.1 g samples of whole venom were dissolved in 1 ml of distilled water and mixed by vortex before application to the column. Gel filtration and the absorbance measurements were kept at the same conditions for the protein standards. Absorbance peaks were pooled and then lyophilized on a Virtis lyophilizer. These fractions were stored at $0^{o}C$ until further use.

Dry CM Sephadex was allowed to swell in 5 times the final bed volume of water and stored at $4^{o}C$ for 24 hours. Excess water was decanted and the gel was packed in a 2.5 x 90 cm glass column. The gel was equilibrated by washing with two times the column volume of 0.1 M KCl, 0.05 M Tris buffer, pH 9.0. Venom fractions were eluted with 50 ml of 0.1 M KCl, 0.05 M Tris buffer pH 9.0, then 50 ml of 0.5 M KCl, 0.05 M Tris buffer pH 10.0, and finally with 200 ml of 0.75 M KCl, 0.05 M Tris buffer pH 10.4. Absorbance measurements were determined as with the Sephadex G-100 column.

Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis was performed on the venom fractions using the pH 8.9 system of Davis (25). This procedure was modified by diluting the acrylamide to 7.5%. Milligram quantities of

the venom fractions were added to the stacking gel, and a current of 2 mamps per gel was supplied until the tracking dye (bromophenol blue) passed into the separating gel. The running current was increased to 4 mamps per gel until the dye was 1 cm from the end of the gels. The gels were stained in aniline blue black and destained in 7% acetic acid.

SDS acrylamide gels were used for MW determinations according to the method of Weber and Osborn (26). The gels contained 1% SDS with a buffer 7.2. Protein standards and venom fractions were loaded onto the gels in mg/ml solutions and electrophoresis was set at 8 mamps per gel. The gels were stained in Coomassie blue and destained with a 25:10:65 solution of 2-propanol-acetic acid-water. The relative mobility for each band was calculated and compared with that of the known proteins.

Toxicity Assays

Vanom components were tested for their lethality in white mice by intraperitoneal injections. The sample solutions were made up at 2 to 5 times the LD_{50} value of whole venom in mice, and 0.15 ml of these solutions was used for injection. Two mice were used for each venom fraction. The mice were checked for vital signs for 24 hours after injection. If still alive after 24 hours, the mice were sacrificed using ethyl ether and the peritoneal cavity was examined for signs of tissue damage.

Amino Acid Determination

Venom toxic factors were analyzed for amino acid composition at the nanomole level using a narrow bore column (27). One hundred

microgram samples were hydrolyzed in 200 µl of 6 N HCl within evacuated sealed test tubes and heated at 110°C for 24, 48 and 72 hours intervals. The dried samples were resuspended to a volume of 2.5 ml and 0.1 aliquots were used for analysis.

Hemorrhagic Activity

The ability of whole and fractionated venom to induce hemorrhage in mice was tested using the method of Ownby et al. (28). Samples were prepared to a concentration of 0.6 mg/ml and 0.1 ml were injected intramuscularly into the thigh of the mice. After 24 hours, the mice were killed and muscle tissue excised. Primary fixation of the tissue was done in 2% gluteraldehyde in 0.27 M cacodylate buffer, pH 7.4 for 1 hour at 4°C. The tissue was then washed in cacodylate buffer containing sucrose. Secondary fixation was done in 1% OsO_4 in cacodylate buffer pH 7.4 for 1 hour at 4°C. The tissue was dehydrated in increasing concentrations of ethanol, and then placed in propylene oxide for two 15 minute changes. This was followed by a 1:1 solution of propylene oxide and polybed 812. Next, the tissue was embedded in polybed 812 using BEEM capsules. Blocks were sectioned on an LKB ultratome L and stained with toluidine blue. Evidence of tissue damage was seen using a light microscope.

Precipitin Tests

Whole venom and fractions were tested for immunologic precipitation with polyvalent antivenin using the method of Oudin (29). Agar concentration was prepared to 0.75% with 0.85 g of NaCl in 100 ml of water for ionic mobility. Venom concentration was kept at 0.6 mg/ml while the

antivenin was prepared according to the directions of the Instituto Clodomiro Picado. Two drops of the solutions were added to the agar wells, the plates were sealed with tape and then stored at 37°C in a humid incubator for 24 hours. Precipitin bands between the gels were recorded by hand on notebook paper.

CHAPTER IV

RESULTS AND DISCUSSION

Venom Fractionation

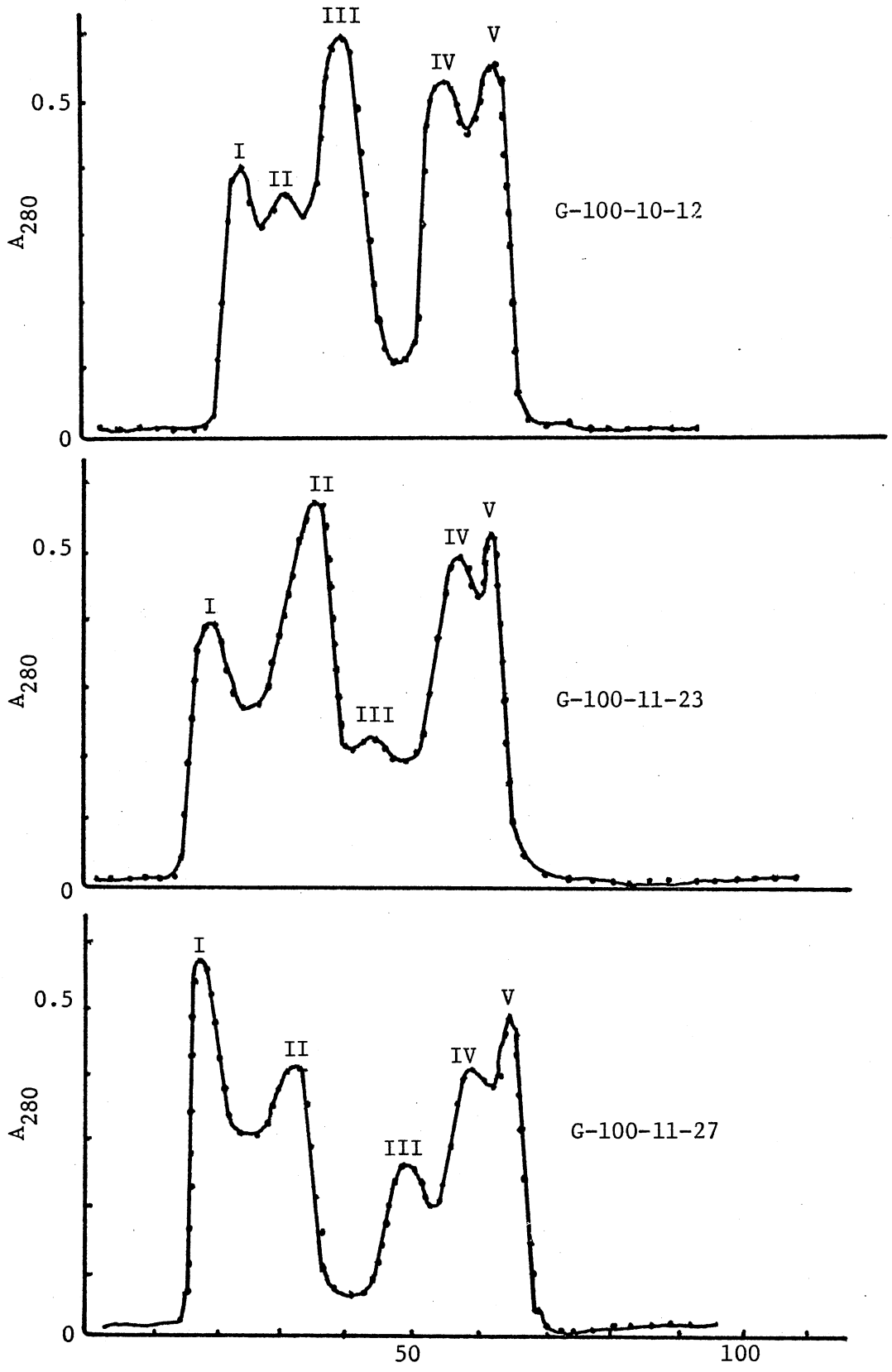
In preliminary work from this laboratory, it was found that the use of buffers in the Sephadex gel filtration of L. muta venom with the subsequent required dialysis and lyophilization steps resulted in the loss of toxic activity. If the dialysis step is eliminated by the use of deionized water as eluent, the toxicity of the fractions is retained. This procedure was used to separate the components of L. muta venom into 5 protein fractions (G-100) as shown in Figure 2.

Each of these venom fractions was concentrated by lyophilization and passed separately through the Sephadex G-100 column again, but there was no further resolution of components. An estimate of the molecular weight range for each fraction was made by comparison with the elution of known protein markers. The calculations were performed on a hand held calculator fitted with linear regression, and are shown in Table II.

In each of the Sephadex profiles, fraction I eluted with the void volume and is assumed to be equal to or in excess of 100,000 daltons. This result is in agreement with Yangs fractionation of snake venoms by Sephadex gel (30). He found that the Crotalidae venoms had elution patterns located closer to the void volume than the venoms of other

Figure 2. Sephadex G-100 Elution Profiles of L. muta Venom. (Eluent: deionized water, 3 ml/tube) Column size: 2.5 x 90 cm.

Protein Markers	MW	Ve (ml)
Blue dextran	2.0×10^6	51 = Vo
Bovine serum albumin	6.7×10^4	132
Ovalbumin	4.5×10^4	159
Chymotrypsinogen A	2.5×10^4	174
Cytochrome C	1.24×10^4	195


III
V
IV
0.5
I
II
A280
G-100-10-12
0
II
V
IV
0.5
I
III
A280
G-100-11-23
0
I
0.5
II
IV
V
III
A280
G-100-11-27
0
50
100
Tube Number

families (30). The remaining L. muta fractions were in the molecular weight range of 100,000 to 3,000 daltons.

TABLE II

ESTIMATED MOLECULAR WEIGHTS OF VENOM COMPONENTS*

	G-100-10-12	G-100-10-23	G-100-11-27
I	> 100,000	> 100,000	> 100,000
II	120,000	100,000	104,000
III	74,500	58,500	54,500
IV	34,000	26,000	24,600
V	4,600	3,600	2,800

*Estimates were made using gel filtration data and are expressed in daltons. Venom used for each column came from the same source.

The toxicity of the venom fractions to mice was tested by intraperitoneal injection at concentrations estimated to be greater than the lethal dose of whole venom. In each test, 2 mice per fraction were marked and observed for 24 hours after injection. The results for 3 gel fractionations can be seen in Table III. Fractions I, II and III were all toxic to the mice but at different rates.

Fraction I caused the most rapid deaths, which occurred within 60 minutes after injection. This was followed by fraction III, which caused death within 2 hours, and then fraction II, fatal in 3 to 6 hours. In each case of fatality, the mice would exhibit uncontrolled muscular spasms, arched spinal columns, difficulty in walking, labored

breathing and would retreat to one corner of their cage. Examinations of the peritoneal cavity after death revealed the presence of blood in the cavity as well as signs of hemorrhage in the internal organs.

TABLE III

OBSERVATIONS OF FATALITY IN MICE DUE TO VENOM FRACTIONS

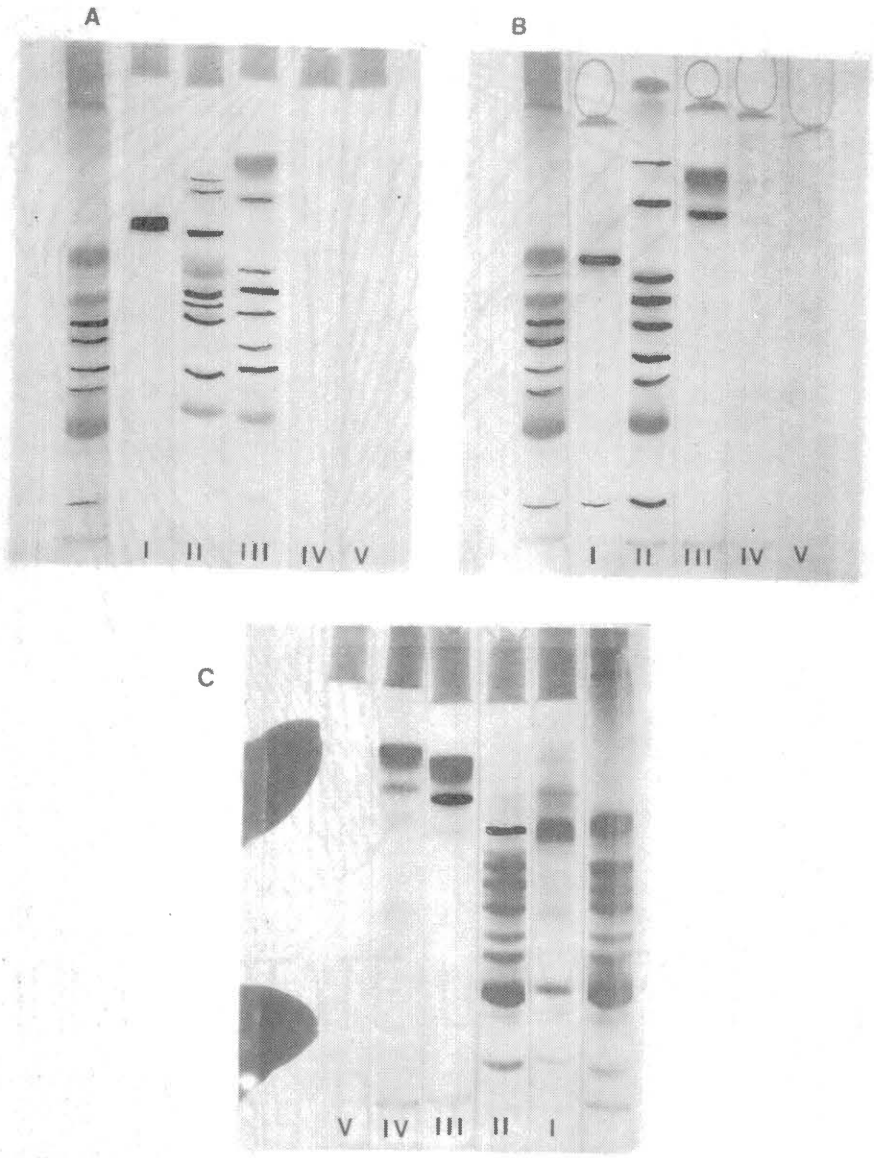
		30 min	1 hr	2 hr	3 hr	6 hr	24 hr
Whole Venom		1/2	2/2	-	-	-	-
G-100-10-12	I	0/2	0/2	0/2	0/2	0/2	0/2
	II	0/2	0/2	0/2	0/2	0/2	0/2
	III	1/2	1/2	1/2	2/2	-	-
	IV	0/2	0/2	0/2	0/2	0/2	0/2
	V	0/2	0/2	0/2	0/2	0/2	0/2
G-100-11-23	I	1/2	2/2	-	-	-	-
	II	0/2	0/2	0/2	1/2	2/2	-
	III	0/2	1/2	2/2	-	-	-
	IV	0/2	0/2	0/2	0/2	0/2	0/2
	V	0/2	0/2	0/2	0/2	0/2	0/2
G-100-11-27	I	0/2	2/2	-	-	-	-
	II	0/2	0/2	1/2	2/2	-	-
	III	0/2	1/2	2/2	-	-	-
	IV	0/2	0/2	0/2	0/2	0/2	0/2
	V	0/2	0/2	0/2	0/2	0/2	0/2

Numbers indicate number of dead mice/number of mice injected.

The number of components in each of the 3 toxic fractions I, II and III was examined by polyacrylamide gel electrophoresis. Figure 3 shows the variation of protein bands detected by this method. Fraction

Figure 3. Polyacrylamide Gel Electrophoresis of Sephadex G-100 Venom Fractions

A. G-100-10-12 whole venom, I, II, III, IV, V
B. G-100-11-23 whole venom, I, II, III, IV, V
C. G-100-11-27 V, IV, III, II, I, whole venom


A
I II III IV V
B
I II III IV V
C
V IV III II I

I contained only one major band. This suggests that a relatively high molecular weight toxin was isolated from L. muta venom as fraction I.

Fractions II and III contain a number of bands that are also present in whole venom. Since both of these fractions exhibit toxicity in mice, and since fraction II appears to contain the 2 electrophoretic bands found in fraction III, the difference in toxicity may be due to the difference in quantity of a single toxin found in both fractions. Fraction III from the G-100-10-12 column was resuspended in 0.05 M Tris buffer and added to a 2.5 x 90 cm CM Sephadex column. The components were eluted with increasing concentrations of KCl to form 2 distinct peaks as shown in Figure 4. These peaks were concentrated by lyophilization without dialysis, and the residue was resuspended in deionized water to approximate the LD_{50} for whole venom. However upon ip injection, neither of these CMS peaks were toxic to mice. This loss of toxicity may have been due to the separation of toxic factors in fraction III, but could also result from the accumulation of salt in the residue due to the use of Tris buffers as eluent. This separation was attempted with another fraction III sample, but was unsuccessful.

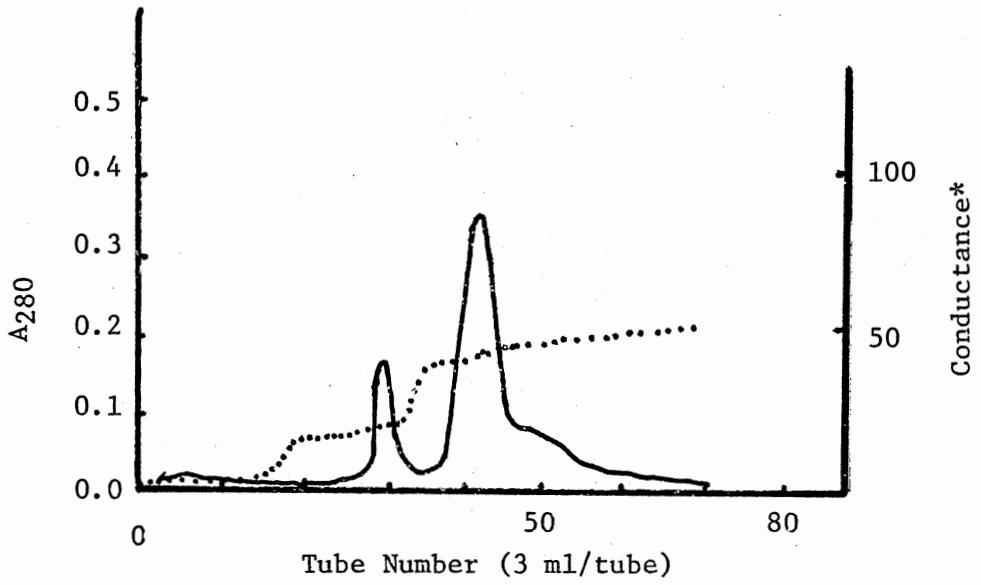
Since fraction III contains only 2 electrophoretic bands that appear to be inseparable without loss of toxicity, this fraction was used as a possible toxin for characterization studies. Because of the inexact reproducibility of the Sephadex profiles, a better method would have been to pool all the fraction III samples from many columns and then attempt better resolution.

Molecular Weight Determination

The molecular weights for fractions G-100-11-23 I and G-100-11-27

Figure 4. CM Sephadex Elution Profile for G-100-10-12 III

Eluent: 50 ml 0.05 M Tris, 0.1 M KCl pH 9.0
50 ml 0.05 M Tris, 0.5 M KCl pH 10.0
200 ml 0.05 M Tris, 0.75 M KCl pH 10.4



*Measured in milliMho

III were derived by SDS polyacrylamide gel electrophoresis. The relative mobility of these fractions can be seen in Figure 5 compared to that for proteins of known molecular weight. Calculations showed that fraction I corresponded to a weight of 139,600 daltons, while fraction III had 2 SDS bands with weights of 62,400 and 20,300 daltons.

These MW values are similar to those obtained from hemorrhagic toxins isolated from other Crotalidae venoms. Ohsaka et al. (31) found 2 hemorrhagic factors in Trimeresurus flavoviridis (Habu) venom. HR1 was an acidic protein with a molecular weight of 104,000 and a pI of 4.3, while HR2 was further resolved into components HR2a and HR2b. All 3 toxins, HR1, HR2a and HR2b, are thought to be specific proteolytic enzymes with unidentified specificity (32). The venom of Agkistrodon halys blomhoffi (Mamushi) also contains 2 hemorrhagic factors. The HR-1 factor was isolated as an acidic glycoprotein with a weight of 80,000 to 90,000 daltons (33). Bjarnason and Tu (34) found 5 hemorrhagic toxins in the venom of Crotalus atrox. The largest of these toxins, designated HT-a, has a weight of 68,000 while the other 4 toxins all have weights of 24,000 to 26,000 daltons.

Amino Acid Analysis

Analyses were obtained for the 24, 48 and 72 hour hydrolysis products of the Sephadex fractions I and III. The averaged results shown in Table IV are expressed in number of residues based on the molecular weights derived by SDS gel electrophoresis. For fraction III, this was assumed to be 59,800 daltons. Also shown is the percent amino acid based on the total amount detected. These values do not account for cysteine or tryptophan which may be present.

Figure 5. SDS Polyacrylamide Gel Electrophoresis of L. muta Toxic Fractions

	Protein Markers	MW	Mobility
1)	Cytochrome C	1.24×10^4	0.698
2)	Chymotrypsinogen A	2.5×10^4	0.492
3)	Bovine Serum Albumin	6.7×10^4	0.357
4)	Lactate Dehydrogenase	1.4×10^5	0.197
5)	G-100-11-23 I		0.201
6)	G-100-11-27 III		0.439, 0.533

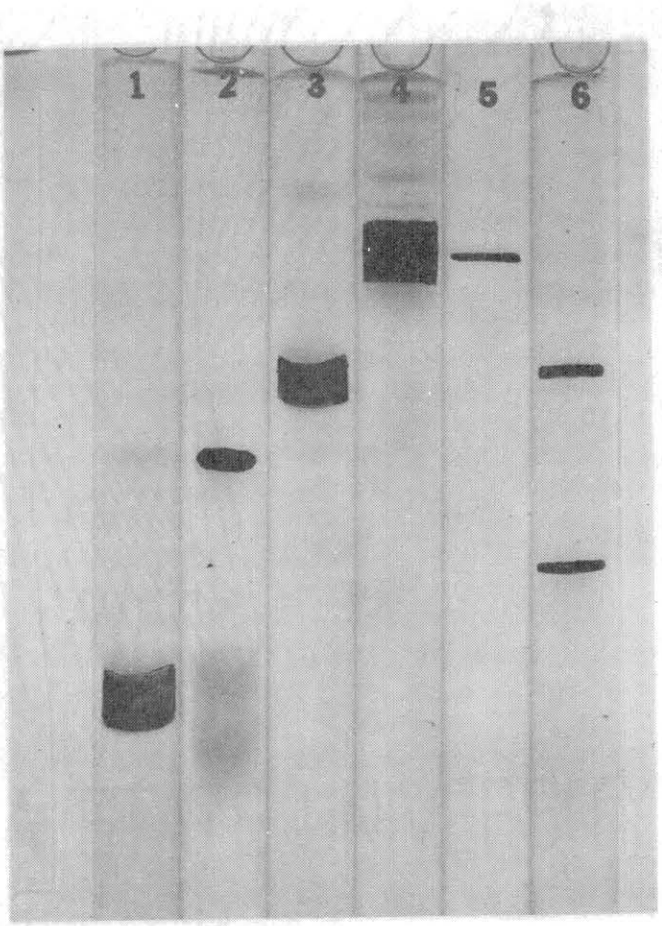

1
2
3
4
5
6

TABLE IV

AMINO ACID COMPOSITION OF L. MUTA TOXIC FRACTIONS

	G-100-11-23 I		G-100-11-27 III	
	Residues	%	Residues	%
Asp	172.8	13.1	72.1	14.1
Thr	73.2	5.6	26.2	5.1
Ser	94.3	7.2	27.4	5.3
Glu	132.9	10.1	68.9	13.4
Pro	64.7	4.9	43.6	8.5
Gly	119.7	9.1	43.7	8.5
Ala	80.5	6.1	37.2	7.3
Val	74.6	5.7	22.1	4.3
Met	22.7	1.7	9.0	1.8
Ile	68.8	5.2	27.6	5.4
Leu	92.9	7.0	36.4	7.1
Tyr	49.1	3.7	8.9	1.7
Phe	49.2	3.7	14.8	2.9
His	35.0	2.7	21.9	4.3
Lys	74.4	5.7	26.9	5.2
Arg	66.4	5.1	26.4	5.1

Number of residues are based on molecular weight derived from SDS data. Percent is calculated from (27).

The L. muta fractions appear to be acidic proteins since aspartic and glutamic acids comprise nearly 25% of the amino acid content. Fraction I also shows a moderate content of serine, glycine, and leucine. Fraction III, although known to contain at least 2 components, has a moderate content of proline, glycine, alanine and leucine. A more complete fractionation of this gel filtration fraction would give a better representation of the amino acid content of L. muta toxins.

Interestingly, the amino acid values presented here are similar to the amino acid compositions for several Crotalid venoms. The HR-1

factor from A. halys blomhoffi venom has a pI of 4.18 and shows a large content of aspartic and glutamic acids, leucine, tyrosine and arginine (33). Hemorrhagic toxins a, c, d and e from C. atrox venom are all slightly acidic with 22 to 26% of the total represented by acidic residues and their amidated counterparts (34).

Hemorrhage

Since the L. muta fractions showed signs of hemorrhage when injected intraperitoneally in mice, the 2 isolated fractions, G-100-11-23 I and G-100-11-27 III, were tested for their hemorrhagic activity by an intramuscular injection into the thigh of the mice. Muscle tissue was excised 24 hours after injection, fixed, embedded, sectioned, stained and then viewed by light microscope. Photomicrographs of the tissue damage appear in Figure 6.

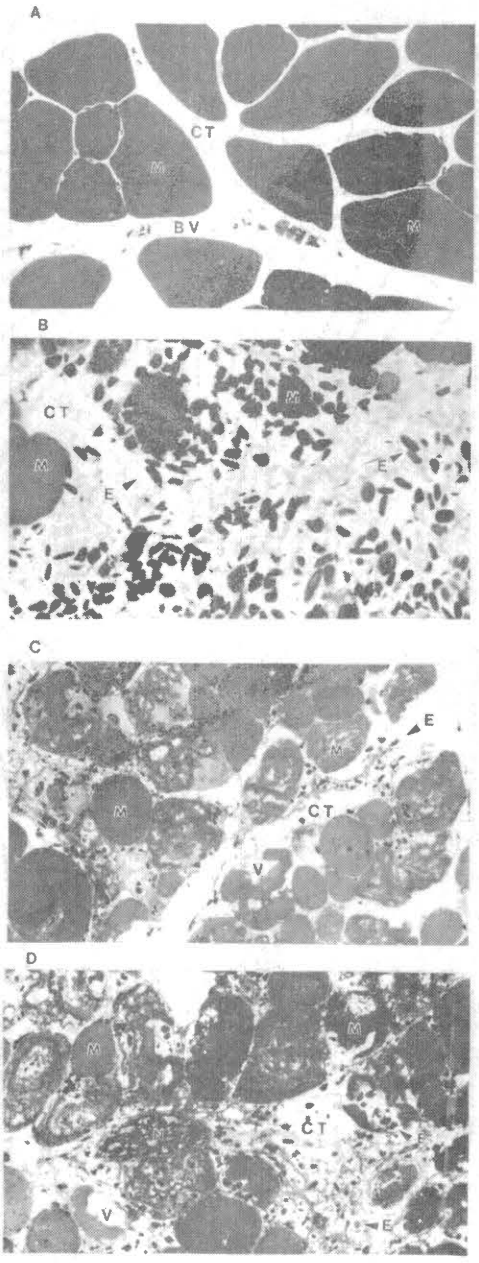
The first picture shows normal muscle tissue taken from the control mice. The muscle cells appear as whole cells and the blood vessels are intact. This is in contrast to the muscle tissue injected with the G-100-11-23 I fraction. Once again the muscle cells look normal, but the presence of numerous erythrocytes indicates the large amount of hemorrhage that has occurred. Also present is what appears to be a smaller number of macrophages in the process of phagocytizing the red blood cells.

The tissue injected with the G-100-11-27 III fraction also shows hemorrhage but the number of erythrocytes present is less than that found in the first fraction. This implies a stronger hemorrhagic ability in fraction G-100-11-23 I, but without a quantitative measure of the hemorrhage this is only a causal observation. Fraction III

Figure 6. Photomicrographs of Tissue Damage Induced by L. muta Toxic Fractions

A. 0.1 ml PSS, 800x
B. 0.1 ml G-100-11-23 I (0.6 mg/ml), 800x
C. 0.1 ml G-100-11-27 III (0.6 mg/ml), 300x
D. 0.1 ml whole venom (0.6 mg/ml), 300x

M: muscle cell	E: erythrocyte
CT: connective tissue	V: vacuole
BV: blood vessel	N: nerve


A
CT
M
BV
M
B
CT
M
E
E
M
CT
C
E
M
M
CT
V
D
M
M
M
CT
E
V
E

III also exhibits slight myonecrotic activity. This is seen in the photomicrographs as the deterioration of some of the muscle cells and the formation of cellular vacuoles.

Finally, the muscle tissue injected with L. muta venom shows both hemorrhage and myonecrosis. Numerous erythrocytes can be seen within the connective tissue as well as within the vacuoles of damaged cells. It appears then, that a major hemorrhagin in the whole venom was isolated as fraction I while fraction III contains a myonecrotic as well as hemorrhagic factor.

Immunodiffusion

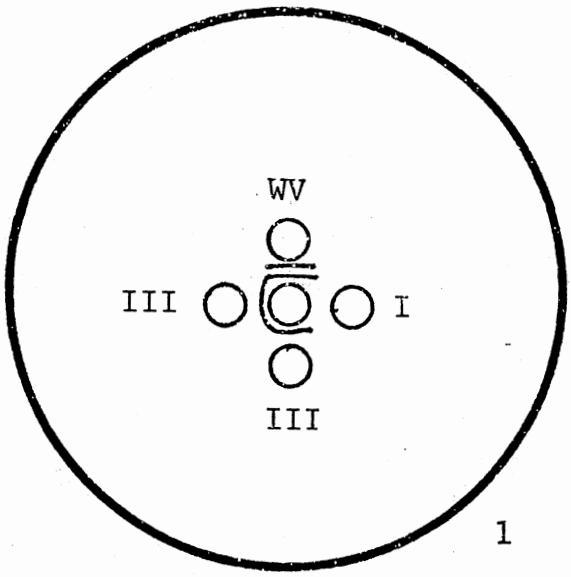
The immunological identity of the toxic fractions of L. muta venom was tested against that of the whole venom. Polyvalent antivenin was placed in the center wells while venom and venom fractions were added to the outer wells. The placement of the toxic fractions was such to allow for lines of precipitation not only between the fractions and antivenin, but also between the fractions and venom. Three Ouchterlony plates with typical results are shown in Figure 7.

The first plate shows 2 precipitin bands between the venom and antivenin, one band between fraction III and antivenin and none between fraction I and antivenin. This pattern is repeated in the other 2 plates and establishes the identity of fraction III with whole venom. The absence of any immunological precipitation between fraction I and antivenin is most likely due to physical factors such as concentration of components and distance between the wells. Yet, even with a 3 to 1 concentration of fraction I to antivenin, no precipitation was seen.

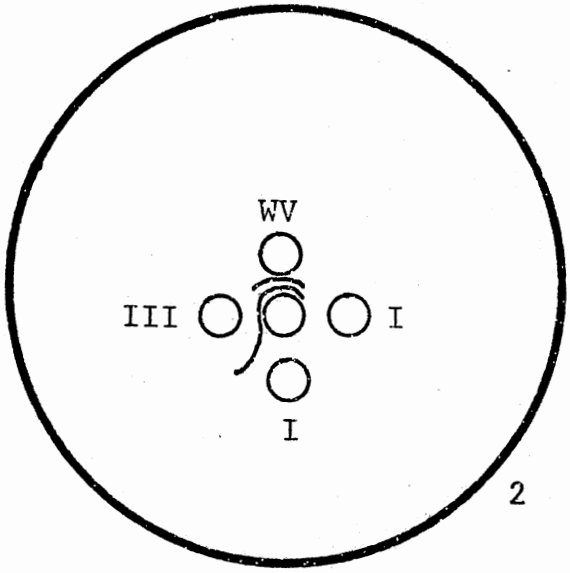
Although Bolaños et al. (35) found no less than 13 immunoelectro-

Figure 7. Ouchterlony Plate of L. muta Toxic Fractions

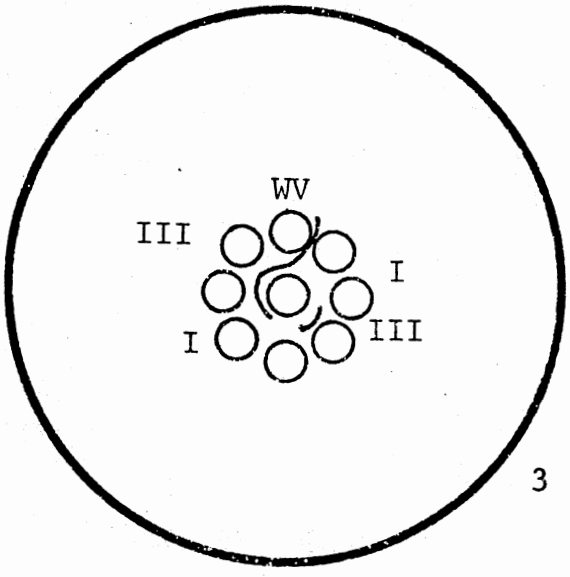
WV: whole venom
I: G-100-11-23 I
III: G-100-11-27 III



1



2



3

phoretic bands between L. muta stenophrys venom and anti-Lachesis serum, the maximum achieved in this study was only 2 bands. Again, physical factors could limit the number of precipitin bands in an Ouchterlony plate, but not to the extent in these results. Additional tests were done increasing the ratio of venom to antivenin to 3:1, but the results were the same.

CHAPTER V

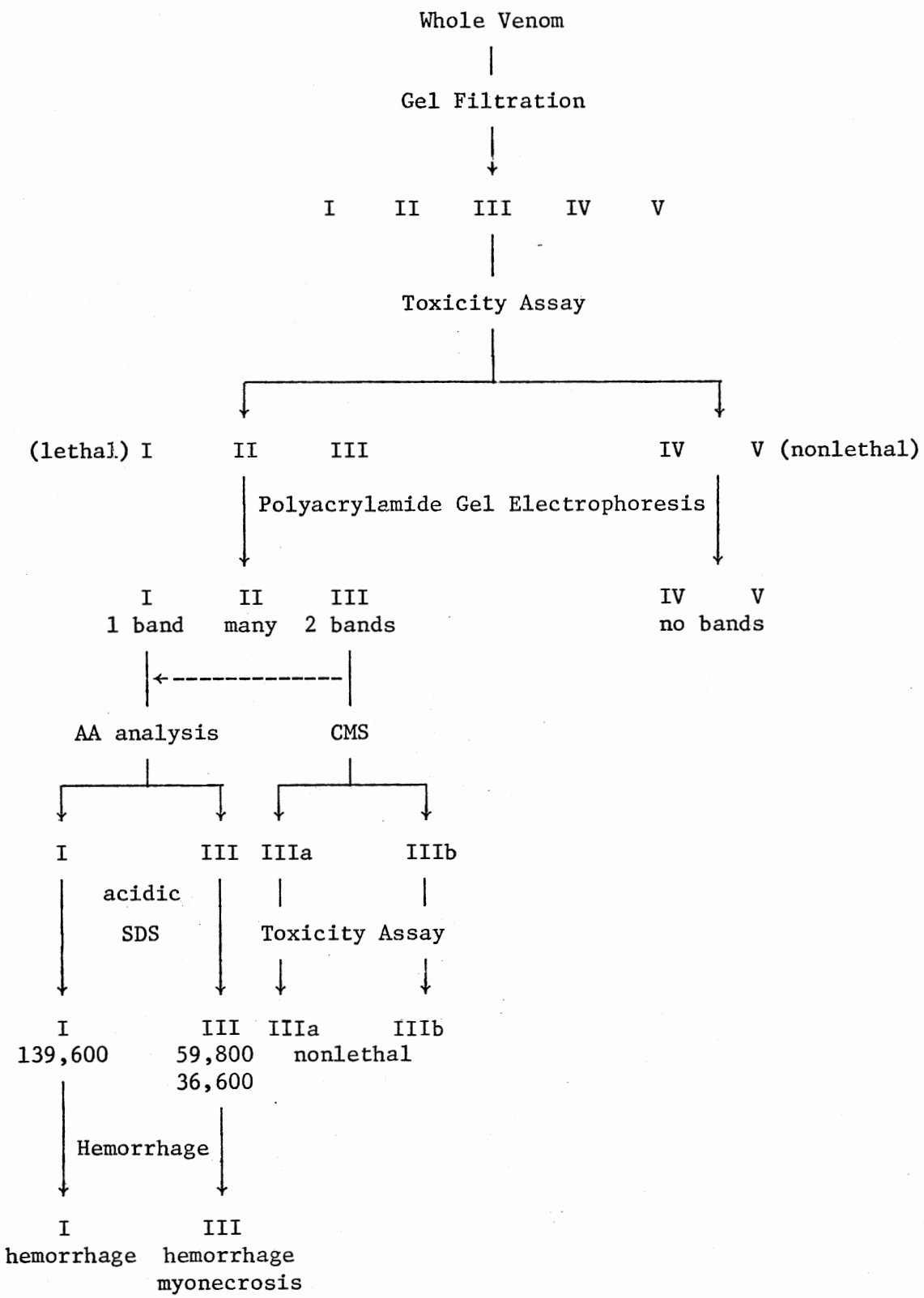
SUMMARY

The fractionation of bushmaster venom raised as many questions as it had hoped to answer. Although this work was preliminary in nature, it still revealed the complexity of biological systems. A schematic diagram illustrating the results of this research is shown in Figure 8.

Gel filtration of the venom produced five distinguishable elution peaks, three of which showed toxic activity. This is the first reported isolation of a toxic fraction from L. muta venom. Fraction I, which eluted with the void volume of the Sephadex column, is a high molecular weight toxin easily separated from the venom. Amino acid composition of this fraction shows an acidic protein of 140,000 daltons which induces hemorrhage in mice. Fraction III contains two electrophoretic bands which appear to be found in fraction II as well. Attempts to separate these bands apparently resulted in loss of toxicity. When subjected to characterization studies, fraction III also showed a large content of acidic residues but induced myonecrosis as well as hemorrhage in mice. The molecular weights for the two fraction III bands were estimated at 60,000 and 20,000 daltons.

With this information in hand, greater investigation of the hemorrhagic factors of L. muta venom can be performed. The role of metallic ions in venom action can be defined and quantitated. Fractions II and III should be subjected to greater analysis so that all toxic

Figure 8. Fractionation Scheme for L. muta Venom


Whole Venom
Gel Filtration
I II III IV V
Toxicity Assay
(lethal) I II III IV V (nonlethal)
Polyacrylamide Gel Electrophoresis
I 1 band
II many
III 2 bands
IV no bands
V
AA analysis
CMS
I
III
IIIa
IIIb
acidic
SDS
Toxicity Assay
I 139,600
III 59,800 36,600
IIIa nonlethal
IIIb
Hemorrhage
I hemorrhage
III hemorrhage myonecrosis

components can be isolated and recognized. A quantitative measure of the tissue damage induced by the toxic factors can then be made.

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